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NSCLC: a target population for a multivalent NY-ESO-1, CT7/MAGE-C1 and CT10/MAGE-C2 vaccine

INAUGURAL-DISSERTATION
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1. Abstract

Background: Lung cancer is a leading cause of cancer death in men and women worldwide. In the past decade efforts were made to improve lung cancer therapy. New chemotherapeutic agents and several targeted drugs have been introduced. But outcome remains poor with an overall five-year relative survival rate less than 20%. Therefore new treatment approaches are needed. Several studies have suggested that standard treatment in combination with immunotherapy could be more effective. Cancer testis (CT) antigens seem to represent promising immune targets and have been tested in multiple vaccine formulations.

Purpose: The aim of this study was to define a non-small cell lung cancer (NSCLC) patient population for a multivalent NY-ESO-1, CT7/MAGE-C1-, and CT10/MAGE-C2 vaccine.

Method: Two consecutively acquired cohorts of NSCLC specimens from chemotherapy naïve or treated patients were collected at the Surgical Pathology in Zurich over the course of 9 years. In a first cohort, consecutive surgically resected NSCLC patients, who have not received chemotherapy yet, were included. In a second, smaller cohort patients who had received chemotherapy were analyzed. Considered histologies were adenocarcinoma, squamous cell carcinoma, and adeno-squamous carcinoma of the lung. A patient database with clinical and pathological characteristics was setup from all collected specimen. Two tumor cores of each specimen were immunohistochemically stained for the CT antigens NY-ESO-1, CT7/MAGE-C1, and CT10/MAGE-C2. Expression frequency and co-expression of the CT antigens as well as the proliferation rate was analyzed. Further the expression of CT antigens was correlated with clinical and pathological characteristics and the CT antigen expression was compared between the chemotherapy naïve and treated patient tumor specimens.

Results: The examined CT antigens were expressed in a coordinated manner. Overall CT10/MAGE-C2 was the most frequently expressed CT antigen. In a subgroup analysis squamous cell carcinomas were found to express NY-ESO-1 at a significantly higher rate

than adenocarcinoma and adeno-squamous carcinomas. Tumors after chemotherapy had significantly higher expression of CT7/MAGE-C1. Further CT antigens tend to be expressed more frequently in tumors with higher pathological grade or proliferation rate.

Conclusion: Based on these results a multivalent vaccine including NY-ESO-1, CT7/MAGE-C1, and CT10/MAGE-C2 could be used in patients with squamous cell carcinoma of the lung after or in conjunction with chemotherapeutic treatment. The detected correlation between the proliferation rate and CT antigen expression leads to the suggestion that CT antigens may play a role in better evaluation of prognosis in the future. As the expression of CT antigens is associated with particular tumor types, a refined selection of patients for future clinical trials could lead to better results.

2. Introduction

Lung cancer is the leading cause of cancer death in men worldwide and women in developed countries. The main risk factor for developing lung cancer is smoking. Eighty percent of all smoking related cancer deaths apply to lung cancers. [1-4].

Non-small cell lung cancer (NSCLC) encompasses various tumors including the two most frequent histologies: 1.) adenocarcinoma and-, 2.) squamous cell carcinoma. Chemotherapy and radiotherapy is used before or after surgery for NSCLC patients diagnosed at early stages. If inoperable, locally advanced or metastatic NSCLC is diagnosed, treatment typically consists of a systemic therapy with platinum-based doublet chemotherapy [5-7]. Patients whose tumor carries an epidermal growth factor receptor (EGFR) mutation may benefit from initial therapy with EGFR tyrosine kinase inhibitors. If the ROS1 mutation status is positive patients can be treated with Crizotinib (tyrosine kinase inhibitor) initially. But the use of tyrosine kinase inhibitors is limited due to side-effects/toxicity of the therapy [8-10].

Despite the introduction of new chemotherapeutic agents and several targeted drugs in the past decade, outcome remains poor with an overall five-year relative survival rate less than 20% (from 4% for advanced up to 54% for localized stages). Only about 15% of lung cancers are diagnosed at early stages. [1, 3] Clearly, new treatment approaches and diagnostic options are needed.

Lung cancer is not typically thought as an “immunogenic” malignancy, but a growing body of evidence suggests that immune responses to lung tumors are present in patients, and their magnitude may correlate with patient outcome [11-13].

The first studies using immunotherapy in lung cancer more than 40 years ago were not successful. This lack of success was commonly explained by the poor immunogenicity of lung cancers. At that time there was probably in general too little knowledge available about immunodeficiency and how to investigate it [14-16]. But even recent studies show a very low objective response rate to lung cancer vaccines (2.6%) [17]. This could be explained by an insufficient number of immune cells capable of recognizing tumor antigens, absent/or

incorrect status of activation of the immune cells at tumor side, or wrong application form. A more direct presentation of antigens to T-cells at tumor side possibly enhances the efficiency of immune reaction and fastens the immunogenic reaction. Macrophage invasion to tumor side is stimulated by hypoxia. Distribution of drugs in hypo-vascularized tumors often is poor. If macrophages could be used as a transport system, the drugs could be applied more targeted [18]. Nevertheless a vaccine would be a very attractive therapy form because opposite to chemotherapy and radiation it allows a more targeted therapy with long term effect by memory cells. Furthermore a vaccine is easy to apply and generally has few side effects [19, 20].

Since the earliest studies knowledge about immunotherapy, tumor environment, and potential target proteins has grown to a greater extent. It is evident that the immune system is able to recognize tumor-specific antigens, which are unique to the tumor, or an overproduction of tumor associated antigens, which can be detected in healthy people as well but are differently expressed in tumors (quantity/time/location) [21, 22]. It is important to take in account that the vaccination induced immune-response targets only the antigens produced at tumor site and can't destroy healthy cells expressing the same antigens and thereby provoke a relevant autoimmune reaction. Further, a mutation in antigen expression by the tumor cells would lead to a loss of target and thus be fatal. Last the vaccine should be broadly available and applicable [21].

The immune-system is able to protect the host from tumor progression up to a certain level. Vice versa the tumor is able to escape from immune response by modifying specific mechanism: in NSCLC for example by producing TGF-b (tumor growth factor) [23], interleukin-10 (probably by inhibiting the production of pro-inflammatory molecules) [24, 25], a down regulation of HLA I (human leukocyte antigen) [26, 27], or a lack of maturation of dendritic cells after capturing dying tumor cells leading to tolerance [22].

The so called "immuno-editing" describes the interaction of tumor- and immune cells in relation to metastatic potential, angiogenesis, invasiveness and matrix degradation and consists of the three phases: elimination (protection), equilibrium and escape (progression)

[21, 28]. For example angiogenesis, which is activated trough a local increase of activity of angiogenic factors, such as interleukin-8 and macrophages, is very important for tumor growth, metastasis and invasion by providing nutrition, oxygen and an exit route for metastasizing cells [18, 29, 30]. Macrophage density at tumor site correlates with interleukin-8 expression, which itself is positively correlated with micro-vessels forming, metastatic potential/distant lymph node metastases and poor prognosis of NSCLC patients [18, 29-31]. As angiogenesis is crucial to cancer growth, efforts are made to develop antibodies to vascular endothelial growth factors (VEGF) [32].

According to Kikuchi et al a significantly decreased number of CD8 positive cells were detected in tumor nests of HLA class I negative tumors, wherefore the immune system fails to recognize tumor cells. This loss of T-cell toxicity is related to poor prognosis in early stages of NSCLC and could be considered as an independent prognostic factor [26].

In the situation of immunogenic allograft rejection an extensive T-cell infiltration can be observed [19]. Several studies have shown that higher tumor infiltration with CD4+ and/or CD8+ T-cells in cancer nests is associated with better survival in NSCLC [12, 27, 33]. Prognosis seems to be affected by the number and distribution of infiltrating immune cells [27, 31]. Interestingly, high CD8+ T-cell infiltration into cancer nests was associated with squamous cell histology [27]. In summary the presence or absence of T-cells in the resected tumor seems to predict more precisely the clinical outcome than tumor stage and nodal status. Therefore the immune response should be taken into account for prognostic evaluation and treatment decision [21].

But where does the activation of specific immune response take place? Bronchus lymphoid tissue (BALT) can normally be observed in fetus and infant lung as well as in inflammatory lung disease. Dieu-Nosjean et al investigated that tertiary lymphoid structures can be found in tumors (tumor induced (Ti-) BALT), which most likely are induced trough a response to tumor micro-environment. Thus the first step in immune response may take place at the tumor site itself, largely mediated by the dendritic cells of the Ti-BALT. Little numbers of

dendritic cells positively correlate with missing Ti-BALT, leading to a decreased number of CD4 cells, poor prognosis, poor T-cell priming and therefore inefficient immunity [34].

Cancer testis (CT) antigens represent promising immunization targets in different types of cancer and have been tested in multiple vaccine formulations [35, 36]. The proteins are silent in healthy adult tissues except in testis, ovary, and placenta without expression of HLA class I, and are aberrantly expressed in cancers. Cancer patients show spontaneous immune responses against various CT antigens making them an ideal target with little potential of autoimmune reaction [37-41]. The expression profile may differ substantially in different ethnical groups [38]. Tajima et al for example report a poor expression of NY-ESO-1 in a Japanese study-population [41]. Further CT antigens are usually expressed in a coordinated manner. Thus a polyvalent vaccine would be preferable in order to increase the eligible number of patients [40-42]. More than 100 CT antigens have been detected but only 19 thereof are capable to generate an immune response [35]. According to Kim et al an expression of at least one CT antigen could be detected in about 73% of lung cancer patients [35]. In various studies the correlation between CT antigen expression, and pTNM stage, grade, cancer histology, gender and age was investigated with divergent results [35, 37, 39, 43, 44]. It seems that CT antigen expression is more frequent in high grade tumors. Therefore CT antigens could play a role in better evaluation of prognosis [37].

The melanoma-associated antigen 3 (MAGE-A3), which is more frequently expressed in squamous cell carcinoma, than in adenocarcinoma [39], has been evaluated in multiple vaccine formulas [45, 46]. MAGE-A3 in combination with an immune-stimulant is currently being evaluated as a vaccine in a randomized, double-blinded, placebo-controlled phase III trial in an adjuvant setting after completely surgical resection of early-stage (IB, II and IIIA) MAGE-A3 positive NSCLC (MAGRIT). Some of this patients received chemotherapy. Whereas only chemotherapy naïve patients with tumor stage IB to II were included in the phase II study. In this smaller study-group no advancement in the disease-free-interval could be found and thereby the primary endpoint was not met [45]. According to the ESMO (European Society for Medical Oncology) congress press release 2014 the results of the

phase III are as well disappointing. No improvement of disease-free survival could be detected in the setting of simultaneous neo-adjuvant chemotherapy administration. The abstract of the study is available on the ESMO website [47].

Another vaccine formulation CV9202 RNActive®, which consists of six different components, among them NY-ESO-1, CT10/MAGE-C2 and CT7/MAGE-C1, is currently being tested in a phase I trial for patients with stage IV NSCLC [48].

Recently evidence emerged that immunotherapy in combination with radio- or chemotherapy is more effective. Different studies illustrated that the expression of cancer testis antigens increases with radiotherapy and through this the immunogenicity of tumors increases as well (γ-Radiation promotes immunological recognition of cancer cells through increased expression of cancer-testis antigens in vitro and in vivo) [14, 22, 49-51]. Demaria et al describe the relation between radiation and immune answer as fallowing: trough radiation the production of “danger” cells, release of pro-inflammatory cytokines (interleukin-1b, TNF-alpha (tumor necrosis factor)), and of prostaglandin E and HLA I increase. A change in tumor microenvironment and vessels was observed as well. The endothelial cells express an adhesion molecule, which is different in tumor and normal vessels and might increase the T-cell invasion at tumor side [22]. Older studies also showed that transferring T-cells after provoked lymph depletion trough radiation is more effective because of a change in quality and impact (for example by elevated production of interleukin-2, TNF-alpha) rather than by their quantity [52]. Recently, antibody-facilitated T-cell induction in cancer was tested. A monoclonal antibody (mAB) against CT antigens was added and the local release was prompted by chemo- or radiotherapy. The complex of mAB and CT antigen was then presented to dendritic cells more easily and leaded to a boosted activation of CD8 T-cells [51].

Due to antigenic heterogeneity and potential for immune escape effective anticancer vaccines should try to include multicomponent and -valent rather than single or oligo-component vaccines and to combine the immunotherapy with agents capable to block factors, which are diminishing the antitumor answer [14, 21]. For example myeloid

suppressor cells (MSC) produced by tumor cells are able to down regulate the elimination of the tumor trough T-cells. One enzyme which is needed for MSC function is arginase I. The tumor produces prostaglandin E2, which itself stimulates the production of arginase I. A therapeutic option could therefore involve a suppression of the arginase I activity with COX-2 inhibitors which then leads to a down regulation of MSC [53].

In summary it seems that vaccination in combination with radio- and/or chemotherapy could be a new treatment approach [54].

In the present study, we investigated the frequencies of expression and co-expression of the three CT antigens, NY-ESO-1, CT7/MAGE-C1, and CT10/MAGE-C2 in a large unselected cohort of consecutive surgically resected NSCLC patients. Further we correlated the expression of CT antigens with clinical and pathological characteristics. In a second study cohort the intensity of CT antigen expression after chemotherapy was observed.

3. Patients and methods

3.1 Data collection

At the Institute of Surgical Pathology Zurich, n=491 consecutive surgically resected NSCLC patients were retrieved from the PathoPro computer files. Data was collected between January 1993 to December 2002 from 7 different referral hospitals (University Hospital Zurich n=397, Stadtspital Waid n=48, Kantonsspital Zug n=18, Spital Männedorf n=12, Spital Uster n=9, Spital Zollikerberg n=6, Klinik im Park n=1). Adenocarcinomas, squamous cell carcinomas and adeno-squamous carcinomas were included. Large cell -, combined small cell - and pleomorphic carcinoma patients were excluded. 17 patients from the first cohort were removed from survival analysis due to lack of data or lethal complications before 30 days post-surgery (n=474). The cut off was set at 30 days post-surgery to exclude all patients with lethal complications not due to tumor progression. In a second cohort 41 patients after treatment with neo-adjuvant chemotherapy were analyzed.

Two cores of each patient were stained. If one core out of a pair was lacking tumor nevertheless data extraction was carried forward.

Progression-free survival (PFS) time was defined as the interval between the date of surgery and disease progression, death or last contact, respectively. Only documented relapses were accepted for warranting progression. During the initial years of data collection a follow-up control with PET-CT (positron emission tomography-computed tomography) was not a standard procedure to document/search tumor relapse. Therefore patients with initial metastatic disease were excluded from PFS calculations (n=432). Overall survival (OS) time was defined as the interval between the date of surgery and death or last contact, respectively. The end date of the follow-up study was 54 months. The mean duration of the follow-up was 43 (range 0-169) and 51 months (range 1-169 months) for PFS and OS, respectively.

3.2 Ethical issues

This study was approved by the ethic committee of the University Hospital Zurich (reference number StV 29-2009 (attached at the appendix)).

3.3 Tissue microarray (TMA) construction

The construction of a set of three TMA's was accomplished with a custom-made, semiautomatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Two tissue cores of 0.6 mm in diameter were taken from each case and transferred into the recipient paraffin block. Wherever applicable in adenocarcinoma, an area representing the solid growth pattern was selected for the first core and a core representing either the glandular, bronchiolo-alveolar, or papillary pattern for the second (n=982). Cores from appropriate control tissue including tonsillar lymphatic parenchyma, normal lung and normal bronchial mucosa were added at the end. Four micrometer thick sections were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, USA).

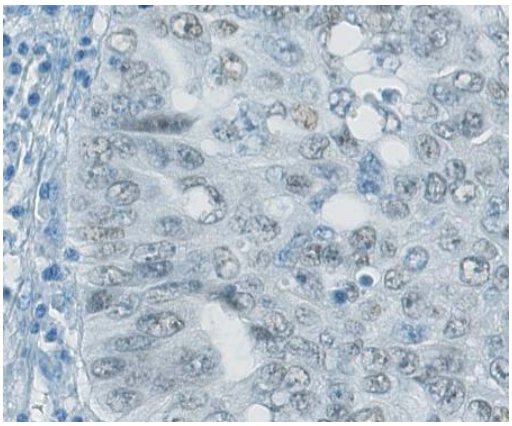
3.4 Immunohistochemistry

The expression of NY-ESO-1, CT7/MAGE-C1 and CT10/MAGE-C2 was analyzed immunohistochemically. Consecutive 3 µm sections were cut from TMA blocks and mounted on glass slides (Super-Frost Plus, Menzel, Braunschweig, Germany). For immunohistochemical staining the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) and Ventana reagents were used. After deparaffinization in xylene, slides were rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase was blocked using Ventana endogenous peroxidase blocking kit after a rinse with distilled water. For antigen retrieval slides were heated with cell conditioning solution (CC1, Ventana) according to manufacturer's instructions. The primary antibody against CT10/MAGE-C2 (rabbit polyclonal, 1:500, ProteinTech Group, Inc.), CT7/MAGE-C1 (clone CT7-33, 1:80, DAKO A/S) and NY-ESO-1 (clone E978, 1:50, ZYMED) were applied adjusted to the Ventana Benchmark system after performing titrations. iVIEW-DAB was used as chromogen. Antibodies were tested on both multi-tissue microassays and whole sections from germ cell tumors as positive control. For negative controls, the primary antibody was omitted. Non-interpretable results, due to lack of target tissue, presence of necrosis or crush artifact, were excluded from analysis.

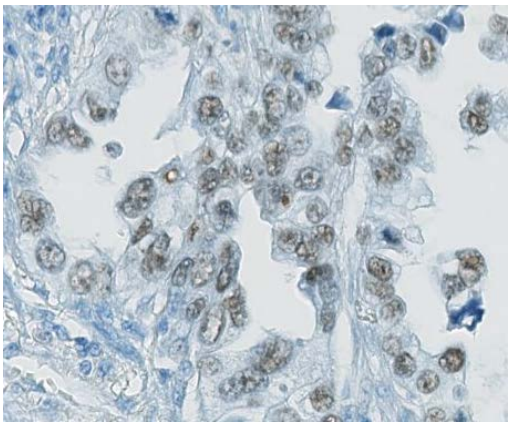
3.5 Interpretation of results

Immuno-reactivity of all antibodies was scored independently by two investigators (investigator one: Prof. Dr. med. A. Soltermann, investigator two: M. Mattle) in a blinded manner. The intensity was semi-quantitatively scored 0 (negative), 1 (weak), 2 (moderate) or 3 (strong) per individual core (figure 1). The percentage of cells having any positivity was proportionally scored 0 (0%), 0.1 (1-9%), 0.5 (10-49%) or 1.0 (50% and more).

1



2



3

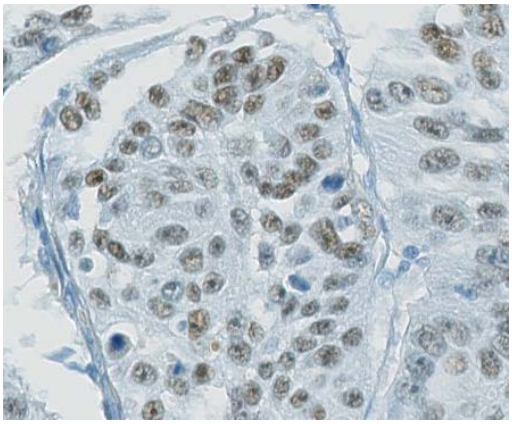


Figure 1: Immunhistochemical fingings for CT10/MAGE-C2 in lung carcinomas.

1. weak, **2.** moderate, **3.** strong.

3.6 Statistical analysis

Data was analyzed by two different methods: First, score results were tested by Chi-squared associations, a test developed to discover whether there is a significant association between two categorical variables. Second, data was analyzed by Kendall's tau-beta correlations, a test developed particularly for small tables and for detecting correlations between non-parametric data. PFS and OS were calculated by the Kaplan-Meier method and differences compared by the log-rank test. The 95% confidence interval (95% CI) was calculated to visualize the uncertainty associated with the estimate (95% CI = mean-value ± (1SE (standard error) x 1.96)). Analyses were carried out using SPSS 22.0 software package (SPSS Inc., Chicago, USA).

4. Results

4.1 Inter-observer variability

Two cores of each tumor were scored independently by two investigators for the presence of NY-ESO-1, CT7/MAGE-C1 and CT10/MAGE-C2 by grading the percentage and intensity. A very low inter-observer variability for the collected results (kappa value: 0.955, 0.935 and 0.989 for CT7/MAGE-C1, CT10/MAGE-C2 and NY-ESO1 respectively) was proved. Thus for better clarity further on only the results of investigator one will be illustrated.

4.2 Cohort und demographic parameters

A total of 491 cases of NSCLC were collected before radio- or chemotherapy. As mentioned above 17 patients were excluded from survival analysis (n=474). While most stage III and IV patients receive chemotherapy or radiotherapy before surgical reevaluation, stage I and II patients are being operated directly, explaining the predominance of this group as shown in table 1. The two main histological groups included where adenocarcinomas and squamous cell carcinomas. The mean tumor size was 3.7cm. The mean age was 64 years, and 30.8% were females (table 1). The mean age and size was used as cut off point for group allocation for OS and PFS calculations. The 4.5-year overall- and progression free survival correlated with the pTNM stage, age and size of the tumor, but not with the gender (table 2 and 3). For example 48% of patients ≤64 years showed a progression free survival compared with 35% of the patients >64 years (table 2).

Table 1: Cohort parameters

n=474	Number	Number%
Age		
≤ 64 y	231	48.7%
> 64 y	243	51.3%
Gender		
Male	328	69.2%
Female	146	30.8%
Histotype		
AC	233	49.2%
SCC	223	47.0%
ASQ	18	3.8%
pT		
T1	98	20.7%
T2	259	54.6%
T3	71	15.0%
T4	46	9.7%
pN		
N0	247	52.1%
N1	143	30.2%
N2	75	15.8%
N3	9	1.9%
pM		
M0	432	91.1%
M1	42	8.9%
Grade		
G1	27	5.7%
G2	244	51.5%
G3	203	42.8%
Size		
≤ 3.7 cm	235	49.6%
> 3.7 cm	239	50.4%

Legend:

AC Adenocarcinoma

SCC Squamous cell carcinoma

ASQ Adenosquamous carcinoma

Table 2: 4.5-year overall survival rates

n=474	4.5y-OS	p-value	95% CI
Age			
≤ 64 y	50%		±6.5%
> 64 y	36%	<0.001	±6.1%
Gender			
Male	40%		±5.5%
Female	49%	0.012	±8.0%
Histotype			
AC	44%		±6.5%
SCC	43%		±6.5%
ASQ	33%	0.477	±17.6%
pT			
T1	61%		±10.0%
T2	45%		±6.1%
T3	37%		±11.2%
T4	9%	<0.001	±8.2%
pN			
N0	54%		±6.3%
N1	36%		±8.0%
N2	25%		±10.0%
N3	0%	<0.001	±0%
pM			
M0	47%		±4.7%
M1	2%	<0.001	±4.7%
Grade			
G1	63%		±18.0%
G2	47%		±6.5%
G3	36%	0.023	±6.7%
Size			
≤ 3.7 cm	53%		±6.5%
> 3.7 cm	34%	<0.001	±6.1%

Legend:

AC Adenocarcinoma

SCC Squamous cell carcinoma

ASQ Adenosquamous carcinoma

OS Overall survival time = interval between the date of surgery and death or last contact.

CI Confidence interval

Table 3: 4.5-year progression-free survival rates

n=432	4.5y-PFS	p-value	95% CI
Age			
≤ 64 y	48%		±6.9%
> 64 y	35%	<0.001	±6.3%
Gender			
Male	39%		±5.7%
Female	46%	0.141	±8.4%
Histotype			
AC	41%		±6.9%
SCC	43%		±6.7%
ASQ	11%	0.008	±18.4%
pT			
T1	61%		±10.2%
T2	41%		±6.3%
T3	34%		±11.6%
T4	5%	<0.001	±6.7%
pN			
N0	50%		±6.5%
N1	35%		±6.7%
N2	23%		±10.4%
N3	0%	<0.001	±0%
pM			
M0	41%		±4.7%
M1	-	-	-
Grade			
G1	52%		±20.0%
G2	45%		±6.7%
G3	34%	0.034	±6.9%
Size			
≤ 3.7 cm	52%		±6.7%
> 3.7 cm	30%	<0.001	±6.3%

Legend:

AC Adenocarcinoma

SCC Squamous cell carcinoma

ASQ Adenosquamous carcinoma

PFS Progression-free survival time = interval between the date of surgery and disease progression, death or last contact. Initial metastatic diseases were excluded.

CI Confidence interval

4.3 Correlation between frequency of protein expression and clinical/ pathological parameters

Two cores of each tumor were stained in this cohort for the presence of NY-ESO-1, CT7/MAGE-C1, and CT10/MAGE-C2. 147/491 patients (29.9%) expressed at least one of the three analyzed CT antigens. The most prevalent cancer testis antigen was CT10/MAGE-C2 with expression in 21% of all cores. CT7/MAGE-C1 was expressed in 16.7% of the cores and NY-ESO-1 in 10.2% (table 4).

Table 4: Frequency and intensity of antigen expression (2 cores of each tumor/patient)

Intensity	**NY-ESO-1**	**CT7**	**CT10**
0	89.8%	83.3%	79%
Core 1			
1	3.1%	4.1%	4.1%
2	3.1%	3.9%	4.9%
3	1.8%	3.7%	4.1%
Core 2			
1	0.6%	1%	1.8%
2	0.6%	1.4%	2.2%
3	1%	2.6%	3.9%

Legend:

0 = no expression

1 = weak, 2 = moderate, 3 = strong

NY-ESO-1 expression frequency was significantly different between histologic subtypes. NY-ESO-1 was significantly higher expressed in squamous cell carcinomas and less frequently in adenocarcinomas or mixed tumor types ($p < 0.001$). This expression pattern was not found for CT10/MAGE-C2 or CT7/MAGE-C1.

In addition CT antigen expression generally correlated with grade by univariate analysis for CT7/MAGE-C1 and CT10/MAGE-C2 (p-value: 0.021 and 0.109 for CT7/MAGE-C1 and CT10/MAGE-C2 respectively), but not for NY-ESO-1 (table 5). When the patients where

stratified according to histology as well a correlation between NY-ESO-1 expression and grade was observed.

Table 5: CT antigen expression correlated with clinical/pathological parameters

n=491		NY-ESO-1		CT7		CT10	
		Chi	**Kendall**	**Chi**	**Kendall**	**Chi**	**Kendall**
Age	p	0.480	0.385	0.630	0.102	0.308	0.130
≤/> 64	t		-0.039		-0.071		-0.066
Gender	p	0.204	0.083	0.174	0.827	0.436	0.436
M/F	t		-0.070		0.010		0.034
Histo	p	0.002	0.000	0.053	0.099	0.501	0.803
A-S-Q	t		0.158		0.073		0.011
pT	p	0.668	0.282	0.607	0.734	0.668	0.702
	t		-0.043		0.013		0.015
pN	p	0.972	0.479	0.812	0.026	0.704	0.297
0-3	t		-0.028		-0.086		-0.041
pM1	p	0.328	0.804	0.861	0.487	0.217	0.315
0-1	t		0.011		0.033		0.047
Grade	p	0.158	0.013	0.021	0.000	0.109	0.000
	t		0.107		0.172		0.154
Size	p	0.071	0.031	0.138	0.030	0.122	0.203
≤/> 3.7	t		-0.095		0.094		0.055

Underlined values are statistic significant

Legend:

p	p-value
t	Kendall's tau-beta correlation value
Chi	Chi-squared associations
Kendall	Kendall's tau beta correlations
Histo	Histology
A-S-Q	adenocarcinomas, squamous cell carcinomas, adeno-squamous carcinomas

4.4 Cancer testis (CT) antigen expression and overall survival/progression free survival

We could not find a difference in progression free survival or overall survival between positive or negative groups of CT7/MAGE-C1, CT10/MAGE-C2 or NY-ESO-1 in the selected NSCLC study cohort (table 6, figure 2).

Table 6: Correlation between OS/PFS and CT antigen expression

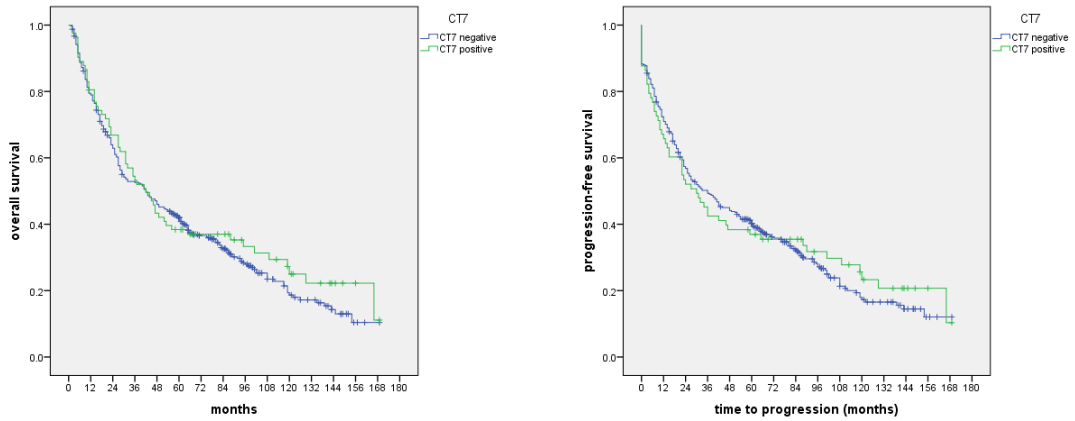
n=432	4.5y-PFS	p-value	95% CI
CT7			
negative	42%		±5.1%
positive	38%	0.774	±11.2%
CT10			
negative	40%		±5.3%
positive	44%	0.251	±10.4%
NY-ESO-1			
negative	41%		±4.9%
positive	44%	0.067	±14.5%

n=474	4.5y-OS	p-value	95% CI
CT7			
negative	44%		±4.9%
positive	40%	0.384	±10.6%
CT10			
negative	44%		±5.1%
positive	41%	0.309	±9.6%
NY-ESO-1			
negative	43%		±4.7%
positive	42%	0.119	±13.7%

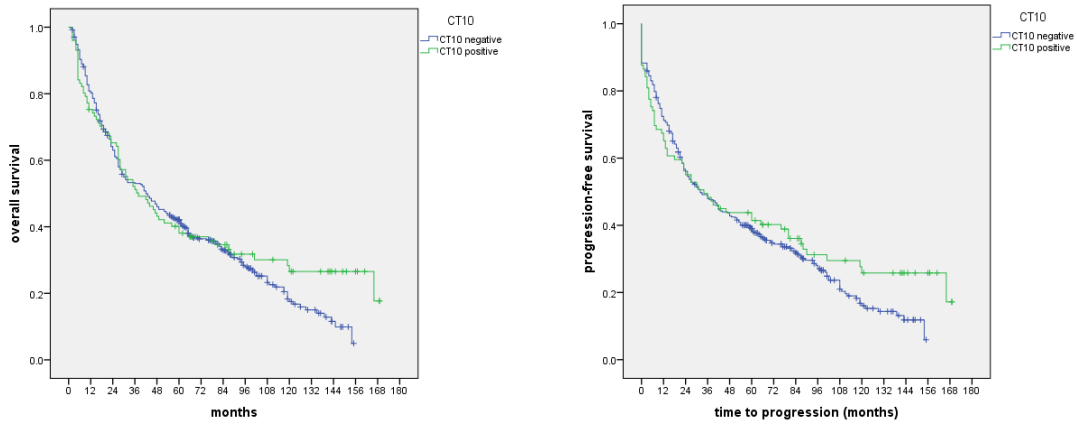
Legend:

CI Confidence interval

a



b



c

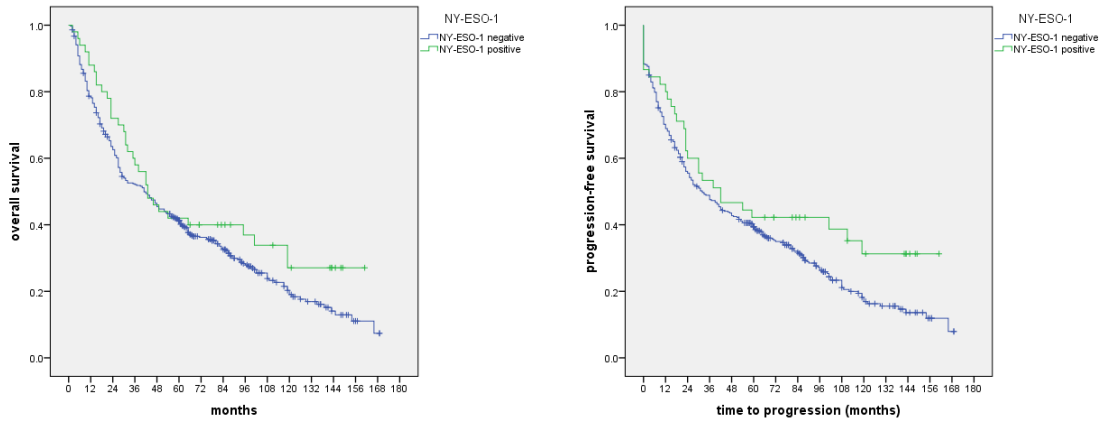


Figure 2: Kaplan-Meier survival curves for overall survival and progression free survival. Comparison between positive and negative groups of **(a)** CT7/MAGE-C1, **(b)** CT10/MAGE-C2, **(c)** NY-ESO-1 in the selected lung cancer patients. Mean duration of the follow-up was 43 (range 0-169) for PFS and 51 months (range 1-169 months) for OS respectively.

4.5 Co-expression of NY-ESO-1, CT7/MAGE-C1 and CT10/MAGE-C2

A significant co-expression was found for CT7/MAGE-C1 with CT10/MAGE-C2, CT7/MAGE-C1 with NY-ESO-1 and CT10/MAGE-C2 with NY-ESO-1 (0.581, 0.227 and 0.199, Kendall tau-beta correlation respectively, p-value < 0.001) (table 7).

Table 7: Co-expressions

			CT7	CT10	NY-ESO-1	MIB-1
Kendall-Tau-b	CT7	Korrelationskoeffizient	1.000	.581**	.227**	.200**
		Sig. (2-seitig)	.	.000	.000	.000
		N	491	490	491	490
	CT10	Korrelationskoeffizient	.581**	1.000	.199**	.148**
		Sig. (2-seitig)	.000	.	.000	.000
		N	490	490	490	489
	NY-ESO-1	Korrelationskoeffizient	.227**	.199**	1.000	.157**
		Sig. (2-seitig)	.000	.000	.	.000
		N	491	490	491	490
	MIB-1	Korrelationskoeffizient	.200**	.148**	.157**	1.000
		Sig. (2-seitig)	.000	.000	.000	.
		N	490	489	490	490

**. Significant correlation

Legend:

MIB-1 KI-67 proliferation index, determined using the MIB-1 antibody for staining (discussed elsewhere).

4.6 Frequency and intensity of CT antigen expression after chemotherapy

Forty-one patients were operated after neo-adjuvant chemotherapy. The chemotherapy consisted of a platinum-based doublet. We found that the expression of CT7/MAGE-C1 was more common in the cohort that had received chemotherapy ($p < 0.001$). As well the intensity of CT7/MAGE-C1 expression was higher after chemotherapy (figure 3). The expression of CT10/MAGE-C2 and NY-ESO-1 was not statistically relevant affected by the use of chemotherapy (p-value: 0.807 and 0.618 for CT10/MAGE-C2 and NY-ESO-1 respectively).

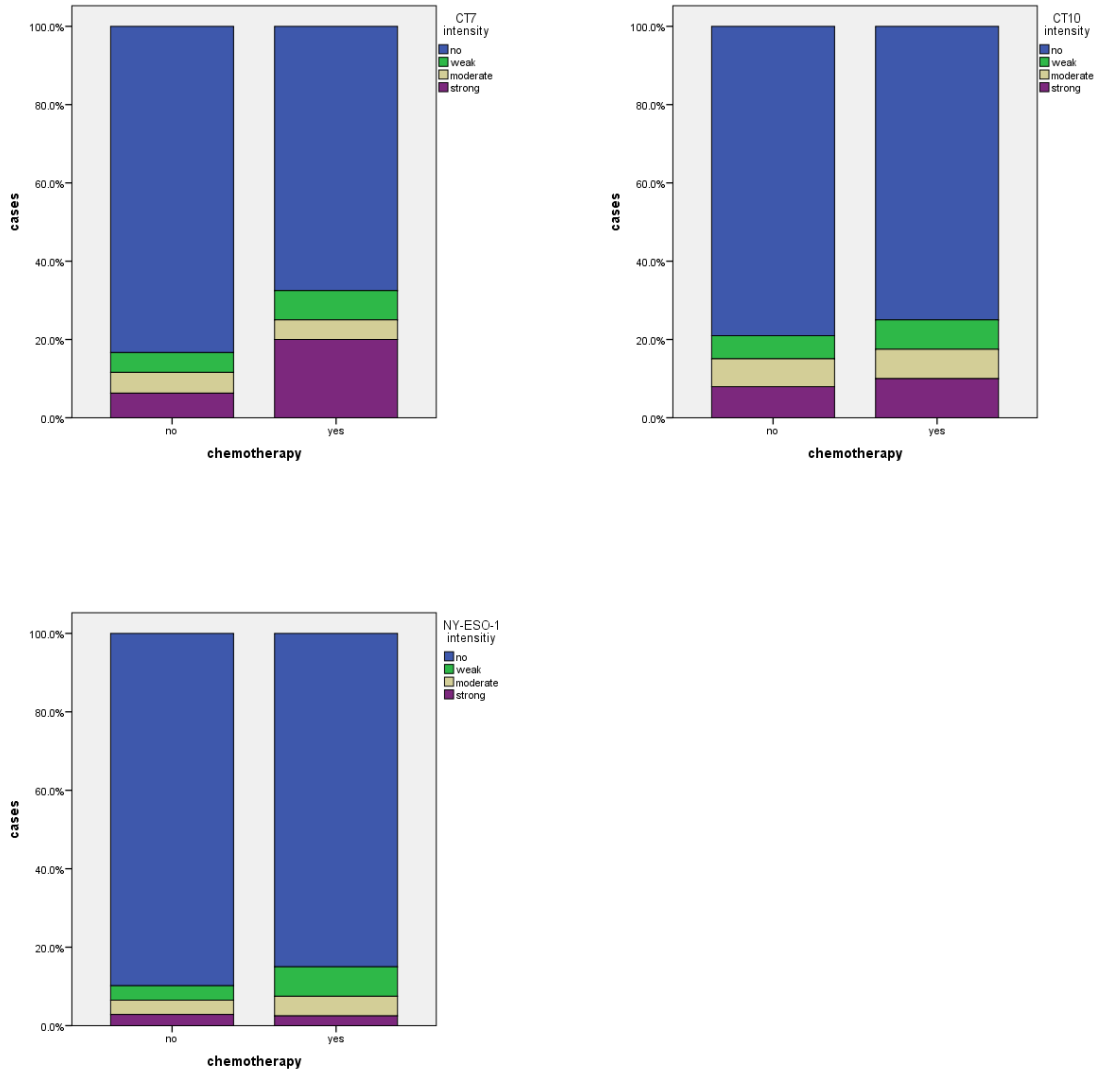


Figure 3: The influence of chemotherapy on CT antigen expression.

4.7 Proliferation rate and expression of the investigated CT antigens

All carcinomas with high CT antigen expression had a high MIB-1 proliferation rate (sum of mib expression of core 1 and 2) (table 7). Carcinomas with low proliferation rates have shown no expression of CT7/MAGE-C1 or NY-ESO-1. In contrast few carcinomas with low proliferation rate did express CT10/MAGE-C2 (figure 4).

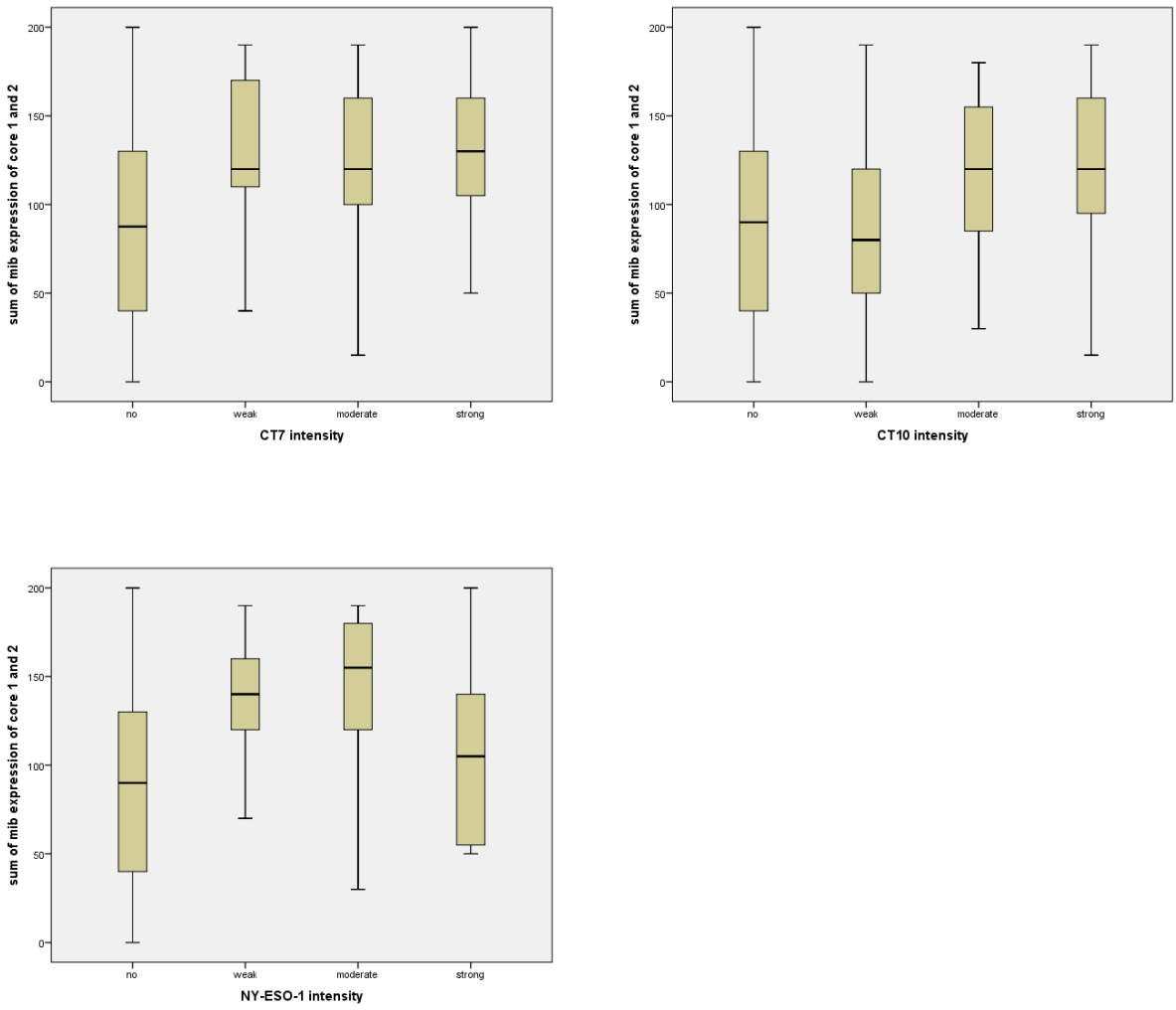


Figure 4: CT antigen expression in relation to the proliferation rate.

5. Discussion

The strength of this study lies within the evaluation of one of the largest series of a NSCLC patient cohort. This allows a rigorous analysis of CT antigen expression in NSCLC subgroups. We chose immunohistochemistry-based analysis due to the clinical relevance of protein expression. We showed that CT10/MAGE-C2 is the most common antigen expressed in NSCLC - compared to NY-ESO-1, and CT7/MAGE-C1 and that the examined CT antigens are expressed in a coordinated manner.

CT antigen can be detected by cytotoxic T-lymphocytes and may be destroyed by them, leading to the suggestion that the expression of CT antigens is linked to a favorable prognosis [39]. Contrary, different studies have demonstrated that the expression of CT antigens has a negative impact on survival and is related with an unfavourable prognosis [39, 42, 44]. In our study we could not observe a correlation between antigen expression and OS or PFS.

The relation between CT antigen expression and clinical and pathological parameters such as pTNM stage, grade, histology, size, gender, and age were investigated several times with divergent results. According to Kim et al a positive correlation exists between age, gender and CT antigen expression, but not with pT and pN Stage or histology [35]. Whereas in a subgroup analysis of MAGE genes a correlation between MAGE-A4 expression, size and pN stage, as well as between MAGE-A3 expression, advanced tumor and poor outcome, plus between MAGE B, -A2, -D2 with pM stage was found [37, 43]. MAGE proteins are linked to cancer progression and metastasis partly by suppressing p53 (inhibition of apoptosis). Targeting of these cells and destroying them could lead to better prognosis [39]. Further Kim et al postulate that NY-ESO-1 is a negative prognostic factor for survival in late stages of lung cancers [38]. We found relevant differences in the expression frequency of CT antigens and histologic subtypes. NY-ESO-1 was more commonly expressed in squamous cell carcinoma and less frequently in adenocarcinomas or mixed tumor types. No correlation was found for CT10/MAGE-C2 and CT7/MAGE-C1. We found no association between age or

gender and CT antigen expression. In accordance with Caballero et al we could illustrate that more aggressive carcinomas, with a high MIB-1 score or a higher pathological grade tended to express CT antigens at a higher frequency [36].

John et al suppose that NY-ESO-1 is a predictive factor for which patients have a potential to benefit from neo-adjuvant chemotherapy [44]. Interestingly, we demonstrated that CT7/MAGE-C1 expression was more common in tumors that had been treated with chemotherapy. No correlation was found for CT10/MAGE-C2 or NY-ESO-1 expression. In previous studies it has been shown that radiotherapy increases the expression of CT antigens *in vitro* on tumor cell lines and tumor explants [14, 22, 49-51]. This study on lung cancer patients suggest that a similar effect could be seen for chemotherapies. If capable of inducing CT antigen expression *in vivo*, treatment with CT antigen targeted immunotherapy could be applied in all chemotherapy and radiotherapy treated lung cancer patients.

In conclusion, the fact that the expression of CT antigens is associated with particular tumor types and patient cohorts allows a refined selection for future clinical trials. CT antigens could not only play a role in immunotherapy but also in better evaluation of prognosis [37]. Because there is a strong co-expression among CT antigens, a multi-CT antigen targeting strategy would be predicted to be most effective. Moreover, it could be argued that CT antigen immunization might be beneficial even to patients with CT antigen -negative tumors, as an induced anti-CT antigen immune response might prevent the emergence of CT antigen expressing tumor cells, thus halting or slowing tumor progression. In squamous cell carcinoma a multivalent vaccine including NY-ESO-1, CT7/MAGE-C1, and CT10/MAGE-C2 could be considered after or in conjunction with treatment with platinum-based doublet chemotherapy.

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8. Curriculum Vitae

Personalien

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Ausbildung

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2010 - 2012	Assistenzärztin Innere Medizin, GZO Spital Wetzikon
2013 - 2014	Assistenzärztin Neurologie, Inselspital Bern
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seit 07/2014	Assistenzärztin Innere Medizin, Universitätsspital Basel
	Rotationen: Intensivstation Universitätsspital Basel, Pneumologie Klinik Barmelweid

9. Appendix

9.1 Ethic votum

Beschlussmitteilung der Ethikkommission SPUK Chirurgie-Anästhesiologie-Pathologie

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Die SPUK Chirurgie-Anästhesiologie-Pathologie hat im Sinne eines Präsidialentscheides das folgende Forschungsprojekt eingehend begutachtet.

Titel des Forschungsprojektes Ref.Nr.: StV 29-2009

StV 29-2009 Rev. (Basisformular vom 01.09.2009) **Biomarker bei Thoraxmalignomen**

Prüfer/in (verantwortliche Studienleiter/in am Versuchsstandort)

Name, Vorname, Titel: Dr. med. Alex Soltermann
Funktion:
Adresse: Institut für Klinische Pathologie, Universitätsspital, Schmelzbergstr. 12, 8091 Zürich

Aktuell beurteilte Unterlagen:

- Basisformular, 01.09.2009
- Studienprotokoll, inklusive Zusammenfassung, 1.9.2009

X normales Verfahren vereinfachtes Verfahren Nachbegutachtung

Die Ethikkommission kommt zu folgendem **Beschluss:**

X **A positiv**
B positiv mit Empfehlungen (siehe Seite 2ff)
C mit Auflagen (siehe Seite 2ff)
Nachbegutachtung durch Ethikkommission notwendig
einfache schriftliche Mitteilung an Ethikkommission ausreichend
D negativ (mit Begründung und Erläuterung für die Neubeurteilung) (siehe Seite 2ff)
E Nicht-Eintreten (mit Begründung) (siehe Seite 2ff)

Der Beschluss gilt auch für die im "Basisformular" gemeldeten weiteren Prüfer/innen im Zuständigkeitsbereich der Ethikkommission.

Auflagen

(erweiterbar)